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Purification and Chemistry of Bacteriophage χ

SYLVIA Z. SCHADE AND JULIUS ADLER
Departments of Biochemistry and Genetics, University of Wisconsin, Madison, Wisconsin 53706

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From a stock of χ phage grown on *Salmonella*, a host-range mutant which attacks *Escherichia coli* was isolated. As in the case of *Salmonella*, only motile strains of *E. coli* are sensitive to χ. The phage shows an eclipse period of 35 min and a minimal latent period of 52 min. The adsorption rate constant is 3×10^{-9} ml/min. Adsorption shows a marked dependence on temperature. Bacteriophage χ was purified by differential centrifugation and CsCl density gradient centrifugation. It contains deoxyribonucleic acid (DNA) which is double-stranded. The DNA has a molecular weight of 42 million and a guanine plus cytosine content of 57%. Of 68 molecules of DNA inspected, 7 were circular. The phage particle weight is about 90 million.

Bacteriophage χ was discovered by Sertic and Boulgakov (27) and was shown by them to attack only strains of *Salmonella* that are flagellated (28). In an extensive study, Meynell (18) showed that, not only must flagella be present, but the flagella must be actively moving in order for infection to take place. The phage attacks also strains of *Serratia marcescens* (10) and *Escherichia coli* (10, and this communication).

Phage χ had never been purified and nothing was known about its chemistry. This paper describes a procedure for the purification of χ from infected *E. coli* and establishes that χ contains double-stranded deoxyribonucleic acid (DNA).

An accompanying paper (23) explores the mechanism by which χ attacks motile bacteria.

MATERIALS AND METHODS

Bacteriophage. Phage χ grown on *S. abortus-equi* NCTC 5727 was obtained from E. W. Meynell. This stock plated with an efficiency of 10^{-3} on a motile strain, AW313, of *E. coli* K-12. A single plaque was picked and used to grow up all subsequent phage stocks. This χ grown on *E. coli* plated half as efficiently on *S. abortus-equi* as on *E. coli*. Otherwise, it had the properties associated with the original χ (18; S. A. London, Ph.D. Thesis, Univ. of Maryland, College Park, 1958).

Bacteria. AW313 was used for all studies except where indicated. AW313 is a derivative of the *E. coli* K-12 strain no. 553 of M. L. Morse, who prepared it from Lederberg strain W2580. A motile isolate of no. 553 was picked from the edge of a swarm on a semisolid tryptone-agar plate. This was treated with phage T1 to isolate a mutant resistant to T1 and T5. Other characteristics of AW313 are F^-, thr^-, leu^-, thi^-, gal_1^-, gal_2^-, lac^-, xyl^-, and ara^-. The flagellar antigen of AW313 was characterized as H48 by the National Communicable Disease Center, Atlanta, Ga. Nonflagellated and paralyzed mutants were isolated from AW313 according to the procedures described previously (1; J. B. Armstrong and J. Adler, Genetics, *in press*).

Media. Tryptone broth, containing 10 g of tryptone (Difco) and 5 g of NaCl per liter of distilled water, was used for growing indicator bacteria and for making dilutions of phage and bacteria. Tryptone-agar, containing 1% agar (Difco) in tryptone broth, was used for streaking bacteria and as bottom layer for phage assays. Semisolid tryptone-agar, containing 0.4% agar in tryptone broth, was used for overlay in χ phage assays.

Phage stocks were grown in Casamino Acids-glycerol, a liquid medium of the following composition: 0.1 M potassium phosphate (*p*H 7), 0.015 M ammonium sulfate, 0.001 M magnesium sulfate, and 10^{-6} M ferric sulfate·$7H_2O$ (11), and 1% Casamino Acids (Difco), 0.2% glycerol, and 1 μg of thiamine per ml.

Phage assay. Indicator cultures were grown in tryptone broth to a concentration of about 2×10^8 bacteria per ml. A 0.1-ml portion of such freshly grown indicator cells was preincubated with 0.1 ml of the appropriately diluted phage suspension for 10 to 20 min at 36 C. Then 2.0 ml of melted semisolid tryptone agar was added to the tube, and the contents were mixed and poured onto petri plates containing about 30 ml of tryptone-agar. Plates were inverted and incubated overnight at 36 C. Plaques were clear, had irregular contours, and measured 0.2 to 2 mm in diameter. It was important that the cultures be in the log phase of growth to obtain highly motile and well-flagellated cells which adsorb χ phage the best. The water content of the agar was critical. The low concentration of agar in the semisolid tryptone-agar overlay provides for motility of the bacteria. The condition of the tryptone-agar underlay was also important. Before use, plates were incubated upright overnight at 36 C, excess moisture was shaken

off the covers, and the plates were stored for 2 to 10 days at 4 C.

Irreversible adsorption, one-step growth, and intracellular growth of χ. Irreversible adsorption was measured by the chloroform technique developed by Fredericq (7). Phage χ was added to 1.5×10^8 bacteria per ml in tryptone broth at a final multiplicity of infection of 0.05 to 0.10 and incubated with rotary shaking at the temperature specified. Every 2 min, 0.1-ml samples were removed and blown into tubes containing 9.9 ml of chilled tryptone broth over 0.5 ml of chloroform. The tubes were mixed with a Vortex vibating mixer three times at 5-min intervals, and then the chloroform was allowed to settle for 10 min. Upon further dilution into chilled tryptone broth, phage were assayed. The initial dilution stops adsorption; the chloroform kills the bacteria. Those phage which are irreversibly attached to the bacteria are lost as plaque-forming centers.

One-step growth experiments were carried out according to the method of Ellis and Delbrück (5). Phage χ was added to 2×10^8 bacteria per ml of tryptone broth at a multiplicity of 0.05. After 20 min of adsorption at 36 C, the cultures were diluted into growth flasks containing tryptone broth at 36 C and were allowed to continue incubating. Samples were removed at about 5-min intervals for determination of infective centers.

Intracellular growth of phage was determined on the same cultures used for one-step growth by diluting samples from the growth flasks through tubes containing chloroform, according to Séchaud and Kellenberger (26). Infected cells lyse in the chloroform tube and release any mature phage, which can then be measured as plaque-formers.

Growth and purification of χ. From a culture of AW313 grown in Casamino Acids-glycerol overnight to saturation (about 10^{10} bacteria per ml), a 100-fold dilution was made into fresh Casamino Acids-glycerol medium and grown to about 2×10^8 organisms per ml. Then four 2-liter flasks containing 200 ml of medium were inoculated with 6×10^6 bacteria per ml of medium and 6×10^5 phage per ml of medium. The flasks were incubated 7 to 8 hr with shaking at 36 C. Lysates with titers of 2×10^{11} to 6×10^{11}/ml were obtained, although no decrease in the optical density of the cultures was observed.

Such lysates of χ were concentrated and partially purified by two cycles of differential centrifugation. A force of $8{,}000 \times g$ for 10 min was used to bring down cells and debris. The supernatant fraction was then subjected to $85{,}000 \times g$ (27,000 rev/min in a Spinco model L rotor no. 30) for 1 hr to pellet the phage. After allowing the pellets to soak in 80 ml of 0.1 M potassium phosphate at *p*H 7 overnight, the phage were resuspended by gentle swirling to disperse the pellets. The centrifugation was repeated, and the second pellets were resuspended as before in 16 ml of the phosphate buffer.

At least 70% of the viable phage was recovered after each sedimentation, even if no precautions were taken to let the pellets soak overnight. Meynell (18) reported the loss of 99.9% viability after sedimentation of the phage at $16{,}000 \times g$ for 90 min, and the loss in viability was correlated with the appearance of large numbers of empty phage. We cannot explain the difference in results.

Solid CsCl was gradually added to the phage suspension over a period of 1 hr to bring the density to 1.48 g/ml. The solution was centrifuged for 10 min at $8{,}000 \times g$, and the precipitate was discarded. The supernatant fraction was centrifuged in three tubes in a Spinco SW39 rotor for 20 hr at 27,000 rev/min to produce a sharp band of phage. Fractions were collected dropwise from the bottom of the tubes. To remove the CsCl, the material containing the main portions of the bands was pooled and dialyzed against 0.1 M potassium phosphate (*p*H 7) or passed through a Sephadex G-200 column. Immediate treatment was necessary since χ phage is unstable in CsCl. All operations were carried out at temperatures between 0 and 4 C.

The purified phage was stored at 4 C in 0.1 M potassium phosphate (*p*H 7). Under this condition, 70% of the phage remained viable at 6 months. The optimal *p*H for storage in potassium phosphate is actually 7.5; here 80% of the phage remained viable at 6 months. The optimal concentration of potassium phosphate was 0.1 M. At 0.01 or 1 M potassium phosphate (*p*H 7), only one-fifth as many phage were viable after storage for 6 months. The loss in viable phage at 0.01 and 1 M was eliminated by the addition of bovine serum albumin at 0.01 mg/ml. Tris(hydroxymethyl)aminomethane buffer, 0.1 M at *p*H 8, allowed χ phage to remain 90% viable with 6 months of storage at 4 C.

DNA isolation. DNA was extracted by a modification of the method of Gierer and Schramm (8) and Mandell and Hershey (16). For all purposes except DNA length measurements, freshly distilled phenol saturated with 0.1 M potassium phosphate (*p*H 7) was used to extract the DNA from a suspension of χ phage at 1.6×10^{12}/ml in 0.1 M potassium phosphate (*p*H 7). Phenol was removed with four washes of ether, and the ether was removed by slowly bubbling nitrogen through the preparation for 4 hr at 0 C.

DNA length measurement. DNA was gently extracted from χ phage in 0.1 M sodium phosphate (*p*H 7) with freshly distilled phenol saturated with 0.1 M sodium phosphate (*p*H 7). It was then dialyzed against 0.01 M sodium phosphate (*p*H 7), mixed with cytochrome *c* and ammonium acetate, and spread onto a water surface by the Kleinschmidt technique (13). The DNA was picked up on grids and shadowed with uranium oxide at an angle of 6 to 10 degrees while rotating the grids. Micrographs of the DNA and of a line-grating replica to determine the magnification were taken on the same day. The length of whole molecules was measured with a map-distance measuring device on prints at a magnification of 22,000 times.

DNA melting curve. For the examination of the melting behavior of χ DNA, the DNA was dialyzed against standard saline citrate buffer (*p*H 7.0) which contains 0.15 M NaCl and 0.015 M sodium citrate. The dialyzed DNA was placed in 3-ml glass-stop-

pered cuvettes, and the optical density was followed at 258 mμ from 25 C to 96.2 C. The temperature was gradually raised over 1 hr to 86 C and then a full hour was allowed for the 86 to 96 C range.

Chemical analyses. Protein was measured by use of the method of Lowry et al. (14). Crystalline bovine serum albumin was used as a standard. Total nitrogen was determined by the micro-Kjeldahl method (31). DNA was estimated with the diphenylamine reagent (3) on trichloroacetic acid precipitates of crude fractions and by direct analysis on purified phage. Deoxyadenosine monophosphate was used as a standard. Ribonucleic acid (RNA) was estimated with the orcinol reagent as described by Schneider (25). 5′-Adenylic acid and deoxyadenosine monophosphate were used as standards. Hexose was determined in two ways, by the cysteine-sulfuric acid method of Dische et al. (4) and by the method of Park and Johnson (19) on an HCl digest of the material.

To check for the presence of RNA in the isolated DNA preparation, the DNA sample was incubated in 0.3 N NaOH for 20 hr at 37 C to release the RNA as nucleotides. The DNA was then precipitated with cold perchloric acid, and the ultraviolet spectrum of the supernatant fraction was measured. The same treatment was applied to a sample of purified ribosomal RNA to verify the completeness of the reaction.

Base composition analysis was made on whole χ phage dialyzed against redistilled water to remove phosphate buffer. According to the procedure of Wyatt and Cohen (34), a sample containing approximately 0.9 mg of χ phage DNA was brought to dryness under a stream of nitrogen. Concentrated formic acid (88%) was added, and the sample was digested at 175 C for 30 min in a sealed test tube. The digest containing the free bases was brought to dryness. HCl (1 N) was added, and a sample was chromatographed on prewashed Whatman no. 1 paper in the isopropanol-HCl-water system. A sample containing adenine, thymine, guanine, and cytosine, a sample of the HCl used as solvent, and a sample of phage T2 DNA digested in the same way were spotted and run on the same chromatogram. Each spot detectable under ultraviolet light was cut out and eluted into 0.1 N HCl. Spectra were measured over the 220- to 300-mμ range, and estimations of base content were made by the differential extinction technique described by Bendich (2).

Sedimentation coefficient. Phage χ was sedimented in 0.1 M potassium phosphate (*p*H 7) at 14,290 rev/min at 6 C in a Spinco analytical ultracentrifuge, with the use of ultraviolet optics to follow the boundary. Photographs were taken at 4-min intervals and measured with a Wilder microprojector.

Filtration with membrane filters. Filtration experiments were carried out on Millipore HA membrane filters (Millipore Corp., Bedford, Mass.) or on Gelman GA membrane filters (Gelman Instrument Co., Ann Arbor, Mich.). The filters were used on either Millipore suction flasks or in Swinny adapter syringe devices, and 1 to 2 ml of phage suspension was filtered.

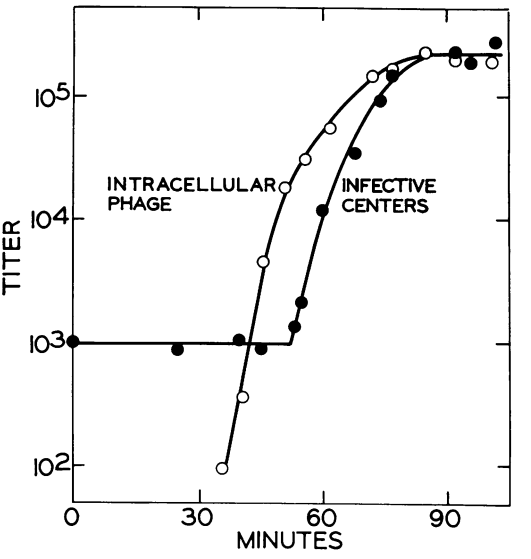

FIG. 1. *One-step growth and intracellular growth of χ on Escherichia coli K-12. The titer reported is the titer of infective centers or intracellular phage in the growth flask. (●) One-step growth: samples were diluted into cold tryptone broth at various times after infection and immediately plated on indicator bacteria to determine the total number of infective centers; (○) Intracellular growth: samples from the same infected culture were lysed with chloroform to release intracellular phage. See Materials and Methods for details.*

RESULTS AND DISCUSSION

Characteristics of the growth of χ. One-step growth and intracellular growth experiments for χ on *E. coli* K-12 (Fig. 1) show that χ has an eclipse period of 35 min and a minimal latent period of 52 min at 36 C. The production of new phage continues up to 90 min, with an average burst size of about 200. Similar results were reported by Meynell (18) for χ grown on *Salmonella*.

A curve showing the adsorption of χ is presented in Fig. 2. The value for the adsorption rate constant, calculated from the initial slope, is 2.5×10^{-9} ml/min. This value ranged from 1.1×10^{-9} to 3.0×10^{-9} ml/min in five determinations. The break in the curve between 8 and 12 min is reproducible. Data for χ adsorption to *Salmonella abortus-equi* indicate adsorption rate constants of 0.26×10^{-9} to 1.3×10^{-9} ml/min (18). These values are similar to the maximal adsorption rates for phage T1 and T2, which are 3.1×10^{-9} and 2.1×10^{-9} ml/min, respectively (21).

Figure 3 is an Arrhenius plot of the adsorption rate constants obtained for χ attachment to

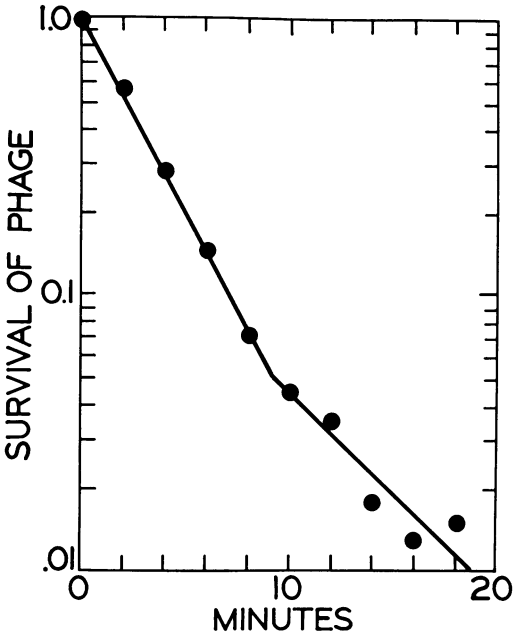

FIG. 2. *Adsorption of χ phage to Escherichia coli K-12. The experiment was carried out at 36 C by use of the chloroform technique described in Materials and Methods.*

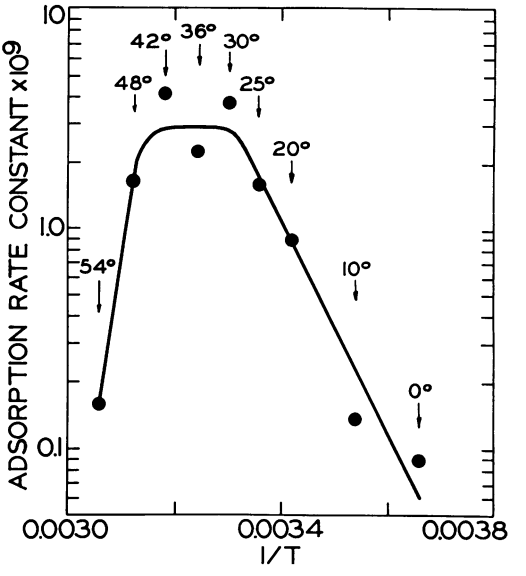

FIG. 3. *Phage χ attachment to Escherichia coli K-12 as a function of temperature. The adsorption rate constants calculated from the initial slopes of adsorption rate curves such as in Fig. 2 are plotted against the reciprocal of the absolute temperature. Before mixing, bacteria and phage were kept 5 min at the temperature indicated.*

E. coli over the temperature range from 0 to 54 C. Each point was calculated from data for the initial slope on an adsorption curve such as in Fig. 2. (At 54 C, the bacteria are rapidly killed; χ adsorption to killed cells was not detected by the chloroform method used here.) The energy of activation calculated from the plot between 0 and 30 C is 22,000 calories. This value is nearly 10 times higher than would be expected if diffusion alone were responsible for the attachment of the phage. For some phage, such as T2 (20) or M13 (30), irreversible adsorption does not show such a high dependence on temperature.

When χ was added at a multiplicity of 10, an *E. coli* bacterial population was reduced to $1/10$ of the original number of viable cells at 40 min and to $1/1{,}000$ at 80 min. This experiment was carried out in tryptone broth at 36 C with log phase *E. coli* at 10^8 bacteria per ml. At 2 hr, the optical density had dropped to two-thirds of its maximal value and was 24% of a parallel uninfected culture. It is clear that χ kills its host and that lysis occurs but is not complete.

Of 267 nonflagellated mutants of *E. coli* tested, 266 were fully resistant to χ phage and only 1 showed slight sensitivity. Of 49 paralyzed mutants of *E. coli*, none showed any sensitivity to χ phage. Of these, 195 of the nonflagellated mutants and 14 of the paralyzed mutants had been selected with χ (Armstrong and Adler, *in press*) and the rest by their failure to swarm (1).

TABLE 1. *Purification and chemical analysis of bacteriophage χ*[a]

Fraction	χ titer (plaques/ml × 10^{12})	Vol (ml)	Recovery (%)	Protein (mg/ml)	DNA (mg/ml)	RNA (mg/ml)	Protein (mg/10^{12} χ)
Lysate	0.55	800	(100)	1.33	0.121	0.15	2.4
8,000 × *g* supernatant fluid	0.55	800	100	0.31	0.058	0.01	0.56
85,000 × *g* pellet	3.6	80	66	1.51	0.41	0.03	0.42
2nd 85,000 × *g* pellet	16	16	59	3.20	1.76	0.14	0.20
CsCl band after dialysis	7.2	16	26	1.30	0.92	<0.02	0.18

[a] The purification procedure and methods used for chemical analyses are described in Materials and Methods.

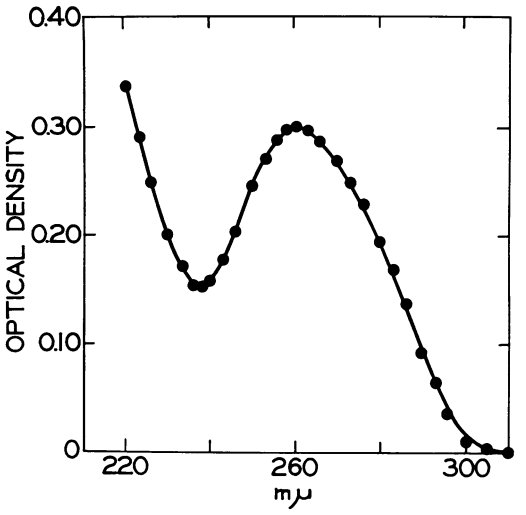

FIG. 4. *Ultraviolet absorption spectrum of purified χ phage. The phage were in 0.1 M potassium phosphate (pH 7). Corrections for light scattering were made according to the method of Englander and Epstein (6).*

These results, which confirm those that Meynell (18) obtained with *Salmonella*, show that χ attacks only motile strains of bacteria.

Purification of χ. The method for purification of χ is described in detail under Materials and Methods. Table 1 shows the results of the purification procedure and, for each fraction, the content of protein, DNA, and RNA. The overall purification was 13.3-fold, based on protein content. The final recovery of phage was 26%. Electron micrographs of the purified phage negatively stained with phosphotungstic acid showed no particles present other than phage. Only an occasional empty phage was found.

Chemical composition of χ. Data on the purified preparation of χ (last line, Table 1) show that χ contains no detectable RNA; rather, χ is a DNA-containing phage whose DNA content is 41%, based on the Dische method (3), and whose protein content is 59%, based on the Lowry method (14). (It is assumed that DNA content plus protein content equals 100%.) Values of 46% DNA and 54% protein were obtained by using the same datum from the Dische method and a total nitrogen analysis (31); these values are considered more reliable, since the value obtained by the Lowry method is based on the assumption that the phage proteins react the same as the standard albumin used.

In a CsCl density gradient, χ phage formed a single sharp band which had a small shoulder on the dense side. The buoyant density of the band was 1.48 g/cc, which according to Weigle et al. (33) indicates a DNA content of about 50% by weight, based on the assumption that the buoyant density for the phage protein is 1.3 [the value for an average protein (32)] and the buoyant density for the phage DNA is 1.72 [the value for a guanine plus cytosine content of 57% (see below; 24)].

The ultraviolet absorption spectrum of the purified phage is presented in Fig. 4. After correction for light scattering, χ phage showed an absorption maximum at 260 mμ and a minimum at 238 mμ.

Properties of the phage DNA. An ultraviolet absorption spectrum of the isolated DNA showed a maximum at 258 mμ, a minimum at 235 mμ, and a 260/280 mμ ratio of 1:95. Chemical analysis showed that it contained no significant amount of protein or RNA.

A melting curve for χ phage DNA is shown in Fig. 5. No increase in optical density was observed below 85 C. The DNA showed a sharp transition, reaching an hyperchromicity value of 38% at 96 C. The melting curve is characteristic of double-stranded DNA. The melting temperature of 92.1 C corresponds to a guanine plus

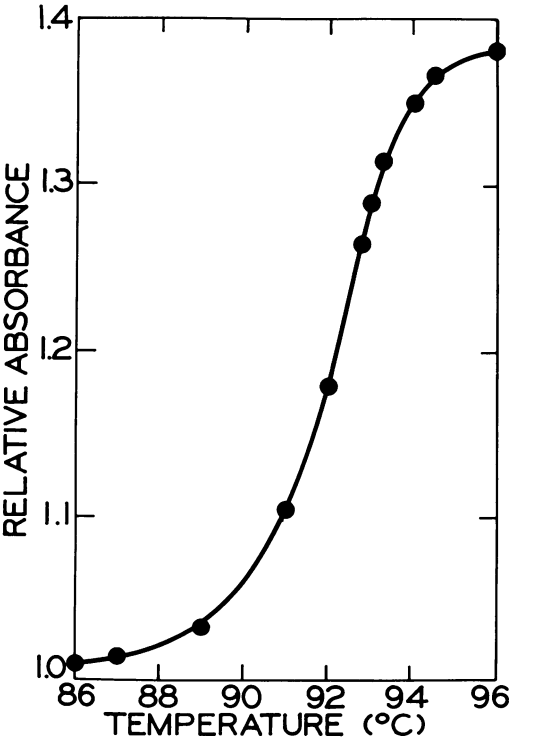

FIG. 5. *Melting curve for χ DNA. Values were corrected for changes in optical density due to thermal expansion. See Materials and Methods for details of the procedure.*

TABLE 2. *Base composition of χ phage DNA*[a]

Component	Mole per cent	
	χ	T2
Adenine	22.4	33.5
Thymine	20.2	30.7
Guanine	28.6	18.2
Cytosine	28.8	
Hydroxymethyl cytosine		17.6
Guanine + cytosine	57.4	
Guanine + hydroxymethyl cytosine		35.8

[a] For details of the procedure, see Materials and Methods.

cytosine content of 55%, based on the curves published by Marmur and Doty (17).

The base composition of χ DNA is presented in Table 2. The concentration of adenine equaled that of thymine, and the concentration of guanine equaled that of cytosine. This is additional evidence that the DNA is double-stranded. The guanine plus cytosine content was 57.4 ± 1.0%. This value is high compared with other *E. coli* phages (24). The guanine plus hydroxymethyl cytosine content for T2 DNA was found to be 35.8%, which is in agreement with 35% reported by Sinsheimer (29).

No evidence was found for unusual bases in χ DNA. There was no ultraviolet-absorbing material on the chromatogram of the DNA digest other than at the positions corresponding to the usual bases. The fact that there was no material between cytosine and thymine at an R_F around 6.2 indicates no detectable uracil or hydroxymethyl uracil, bases found in some phages (12). The spectra determined on each base eluted from the paper in 0.1 N HCl agreed closely with those run on the standard bases, indicating no appreciable quantity of hydroxymethyl cytosine, 5-methylcytosine, or 6-methyl amino purine, whose absorption maxima all differ by 5 mμ or more from the maxima observed (34). Chemical determinations showed no significant amount of hexose, which indicates that χ DNA contains no detectable glucosylated bases.

Of 68 molecules of χ DNA which could be followed along their lengths under the electron microscope, seven molecules were circular. One of the circular molecules is shown in Fig. 6. Unlike the case for λ phage, which also has double-stranded DNA that can be circular (9, 22), there is no evidence for lysogeny by χ.

The lengths of six χ DNA molecules (one circular and five linear taken at random from the above 68) ranged from 21.6 to 21.8 μ. For a molecule 21.7 μ in length, a molecular weight of 41.6 million is obtained when one uses the value 192 daltons per A as the linear density of double-stranded DNA (15).

One can also estimate the phage DNA molecular weight from the chemical analysis of the DNA content per viable phage (last line, Table 1), if one assumes that every phage particle is viable. The estimate is 77 million for the preparation presented in Table 1 and 63 million for another preparation. These estimates are probably high, since it is unlikely that every phage particle is viable. An estimate of the fraction of viable phage is 0.7 for one of the two preparations if 42 million is the actual DNA molecular weight. Since this ratio exceeds 0.5, it is likely that χ carries a single DNA molecule.

Phage particle weight and the size and shape of χ. Based on 42 million for the DNA molecular weight and 46% for the DNA content, the particle weight of χ is 90 million.

Electron micrographs (Fig. 1 and 2 in reference 23) show the shape and size of the χ phage. The phage head is hexagonal in outline and measures 650 A between parallel faces of the hexagon. The tail measures 2,200 × 140 A. These dimensions are in agreement with those reported by Meynell (18).

Filtration on Gelman membrane filters made of cellulose triacetate of 0.45-μ pore size allowed free passage of χ phage. Filters of 0.2-μ pore size allowed passage of 72% of χ phage; 0.1-μ pore size, 0.1%; and 0.05-μ pore size, 0.0%. These

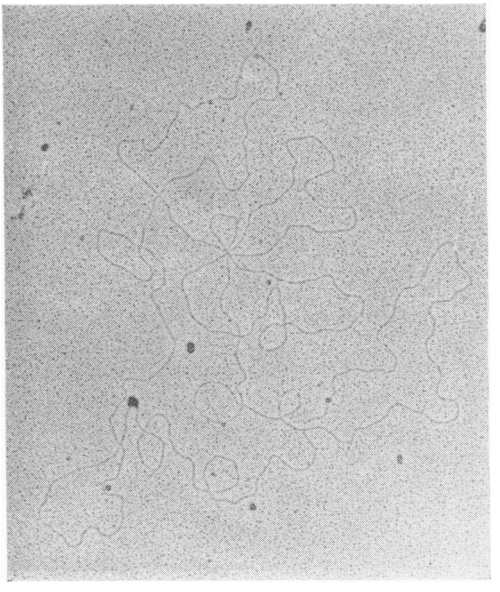

FIG. 6. *Circular DNA molecule from bacteriophage χ. Shadowcast with uranium oxide.* × *27,000.*

results, which indicate that χ phage is larger than 0.05 μ in diameter and smaller than 0.1 μ, are consistent with the dimensions obtained from electron microscopy. Millipore membrane filters made of cellulose nitrate quantitatively adsorbed χ phage at 10^{12}/ml in 0.1 M potassium phosphate (*p*H 7), even when membranes of 0.45 μ pore size were used.

The sedimentation coefficient for χ phage determined in 0.1 M potassium phosphate (*p*H 7) was 262*S*. This sedimentation coefficient for χ is low for its particle weight. Phage λ, which has a somewhat smaller particle weight (about 70 million), has an $S_{20,w}$ of 410 in sucrose (32). Phage λ resembles χ in its shape, but the χ tail is 1.6 times the length of that of λ, and the χ tail fiber (23) is about 10 times the length of that of λ. The low sedimentation coefficient is probably a reflection of the highly asymmetrical shape of χ.

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